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ASSAY OF THEBAINE IN PAPAVER BRACTEATUM [2]

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Second Working Group on Papaver bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This paper was submitted by Dr. E.L. Grew, Research and Development, Macfarlan Smith Ltd.

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ASSAY OF THEBAINE IN P. BRACTEATUM

A simple method for the assay of thebaine in Papaver bracteatum is required in order to cope with the large number of samples arising from a programme of breeding and selection work connected with the attempt to develop an improved variety suitable for the production of thebaine on a commercial basis. For this purpose most of the assays described in the literature are too tedious and time-consuming, requiring in many cases prior separation of thebaine from the other alkaloids of the poppy. Gas-liquid chromatography has been shown to give good results but is also time-consuming. A colorimetric method similar to the Guarino assay for morphine, which can be adapted for use in an AutoAnalyzer, is considered most suitable. Colorimetric assays for thebaine described in the literature depend on conversion of thebaine to the phenolic thebenine which is then determined by a variety of colour reactions based on its phenolic nature. These methods are thus susceptible to the presence of other phenolic materials and do not claim specificity for thebaine. Even when phenols are removed by an ion exchange procedure prior to conversion of thebaine to thebenine, many poppy samples were shown to contain other readily-oxidisable substances which gave colour reactions with phenol reagents.

A differential procedure has been devised whereby the poppy sample is extracted with cold, very dilute acid, two aliquots of the clarified extract taken, the first treated with strong hydrochloric acid and heated for a period sufficient to convert the thebaine to thebenine, the second similarly treated with acid but not heated. Both are then subjected to a colorimetric assay of phenolic material such as that described by Sakurai, J. Pharm. Soc. Japan, 1960, 80, 909, the difference representing the thebaine content of the sample. In this way the effect of initial colour of the sample and also of other substances which give colour reactions with the phenol reagent are eliminated, the only interference being from substances which may be converted to colour-producing substances by the period of heating alone. There is no evidence that such substances are present in the poppy samples tested to an extent sufficient to affect the usefulness of this method as a screening procedure.

Procedure for manual operation

The dry poppy sample is milled through a 20 mesh screen and 0.25 gramme weighed out into a 20 ml vial with a polythene closure. 10 ml of distilled water is added together with 1 ml of normal hydrochloric acid. The vial is then shaken vigorously on a mechanical shaker for 30 minutes and the slurry filtered through Whatman 42 papers without washes. 1 ml of the clear filtrate is pipetted into each of two 20 ml vials and 1 ml of 2N hydrochloric acid added to each. One only of these vials (loosely capped to avoid loss by evaporation) is placed in a hot water bath at 95 to 100°C for 25 minutes, then cooled. To each vial is then added 1.5 ml of 0.5% 4-aminoantipyrene aqueous solution and 0.6 ml of 1% potassium ferricyanide aqueous solution. After shaking for exactly 1 minute, 2 ml of 10% ammonia aqueous solution is added to each and the vials shaken and placed in the hot water for 5 minutes. After cooling, 1 ml of ethanol is added to each vial and the total volume made up to 10 ml with water. The solutions should be clear at this stage but if the concentration of thebaine in the original acid extract is more than 200 mg/L (about 1% in the sample tested) a brown flocculent precipitate may gradually appear, rendering the solution turbid and the result invalid. In such cases, the assay must be repeated after diluting the acid extract by a factor of 2 (for samples containing up to 2% thebaine) or proportionately more for samples of higher thebaine content. If clear, the absorbance of each solution is read at 435 mμ.

The nett absorbance (of the heated sample less that of the unheated sample) is compared with a calibration chart made by carrying out the above procedure using a series of standard solutions of thebaine hydrochloride ranging in thebaine content from 50 to 200 mg per litre in place of the filtered poppy extract. The graph approximates to a straight line through the origin having a slope of about 60 mg/L per 0.1 absorbance. This gives the thebaine content of the poppy extract in mg/L and this figure multiplied by 0.0044 gives the thebaine content of the poppy sample as a percentage. If any absorbance reading exceeds 0.6 or if the nett absorbance exceeds 0.4, the assay should be repeated after diluting the poppy extract by an appropriate factor to reduce the absorbance below this figure, the result being correspondingly corrected. Many samples are found

to give very high 'blank' readings, particularly samples of leaves etc containing chlorophyll and the accuracy of the assay is therefore poorer for those than for samples (of root or capsule for example) giving low 'blanks'. Similarly, low results are more subject to inaccuracies than high ones and figures indicating less than 0.1% thebaine in the poppy should not be taken as significant.

Adaptation for AutoAnalyzer use

For use with an AutoAnalyzer, the method can be considerably streamlined. The first heating stage may be modified from 25 minutes at 100°C to 25 minutes at 80°. The second period of heating may also be reduced to 80° or even omitted altogether. Final filtrations are not usually necessary except with samples of very high absorbance, which should in any case be repeated at a lower concentration. The use of ethanol may be omitted and the concentration of the reagents may be varied to give the correct final volume without final adjustment with water. Results produced by the AutoAnalyzer adaptation of the assay are more reproducible than those obtained by hand, as delays and agitation stages are standardised and, except in the circumstances mentioned above, the absorption readings completed before any turbidity in the final solution has time to develop.

This modified version of the Sakurai assay has been found to give results which are in reasonably good agreement with those obtained by GLC, as shown in the table below:

		% thebaine on dry weight	
Sample	Ref. No.	Modified Sakurai	G.L.C.
Root	1972/5A	0.65	0.66
Root	1972/5B	0.72	0.61
Root	1972/5C	0.55	0.61
Root	1972/6A	0.51	0.59
Root	1972/6B	0.60	0.59
Root	1972/6C	0.56	0.69
Root	1972/7	0.07	0.01
capsule, stem, root	P31973/30 (1A)	0.30	0.30
do	P31973/31 (1A)	0.24	0.28
do	P31973/32 (2B)	0.20	0.18
do	P31973/33 (2B)	0.14	0.14
capsule	P31973/30A	1.6	1.9